AD_____

Award Number: DAMD17-99-1-9142

TITLE: Mechanisms of Growth Factor Attenuation of Cell Death in Chemotherapy Treated Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Carla Van Den Berg, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80262

REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

122068

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual Summary (1 Aug 01 - 31 Jul 02)

4. TITLE AND SUBTITLE
Mechanisms of Growth Factor Attenuation of Cell Death in Chemotherapy Treated Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-99-1-9142

6. AUTHOR(S)
Carla Van Den Berg, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80262

E-Mail: carla.vandenberg@uchsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

20030122 068

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

In previous years of this project we have demonstrated the pro-survival effect of IGF-I through activation of the kinases, phosphatidylinositol (PI)-3 kinase and Akt. Interestingly, IGF-I also potently activates JNK (c-Jun N-terminal kinase) downstream of PI 3-kinase. The biological consequence of IGF-I activation of JNK has been a recent, intense focus of my lab. Recently, we have demonstrated that activation of JNK by IGF-I results in a negative feedback loop in IGF-I signaling. Activated JNK serine phosphorylates IRS-1 (insulin-receptor substrate). Other investigators have recently shown that phosphorylation of Ser307 result inhibits downstream effects of both insulin and IGF-I responses. Further, we are determining at what level in the apoptosis cascade that IGF-I activated Akt inhibits cell death. These preliminary studies indicate that that Akt is working independently of caspase 9, a putative Akt and p53 target.

14. SUBJECT TERMS
breast cancer, IGF-I, chemotherapy, signal transduction, PI 3-kinase, JNK

15. NUMBER OF PAGES
48

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....5

Reportable Outcomes.....5

Conclusions.....5

References.....

Appendices.....6

Introduction

IGF-I receptor (IGF-IR) overexpression is a frequent aberration in found in breast tumors. IGF-IR activation may serve its greatest purpose in cancer cells by activating intracellular PI 3-kinase and its downstream target Akt to convey proliferation and survival of breast cancer cells. In breast cancer cell lines, treatment with IGF-I results in IGF-IR activation and tyrosine phosphorylation of the IRS-1 docking protein. The p85 subunit of PI 3-kinase then binds to IRS-1 to activate downstream kinases such as Akt and $p70^{S6}$ kinase. IGF-IR is an important target for development of new breast cancer interventions since its expression has been observed in 87% of breast cancer specimens. Breast cancer cells expressing IGF-IR undergo a robust proliferative response when exposed to the receptor's ligands. A great amount of research has focused on the effect of growth factor activation of Akt on breast cancer cell survival, with the belief that Akt must convey the majority of PI 3-kinase effects. My lab has studied the survival responses induced by IGF-I treatment of breast cancer cells and how these responses may be altered by cellular stress, including chemotherapy and radiation treatment induction of the p53 tumor suppressor protein.

Body

Research accomplishments: In the past year our efforts to identify the mechanisms of IGF-I pro-survival responses have been separated into two separate projects. Both of these projects stem from the Career Development Award. The first is to understand the mechanism(s) and/or effects of IGF-I activated JNK in breast cancer cells. We are also studying if the outcome of IGF-I activated JNK is p53 dependent. The second project is to understand how IGF-I activated Akt inhibits the apoptosis cascade of proteins. We initially anticipated that Akt would inhibit caspase 9 in a p53-dependent fashion. Our current results indicate otherwise. For purposes of this progress report, our findings will be presented as outlined in the initial Statement of Work of the application.

Task 1 is to describe IGF-IR cytoprotective effects through PI 3-kinase.

In previous progress reports we have demonstrated IGF-I's survival effects through activation of PI 3-kinase and Akt. Overexpression of a constitutively active form of Akt further supports our findings of Akt's survival properties. Last year we were awarded an RO1 grant to study the effects of IGF-I activation of p53 mediated apoptosis. We anticipated that Akt would inhibit p53-mediated apoptosis by phosphorylation of caspase 9. In the past year our data suggest that for some forms of DNA damage, we believe that Akt is working independently of caspase 9. Now we are trying to determine the point of apoptosis inhibition by Akt.

Task 2 is to characterize the potential interactions between the PI 3-kinase survival and stress-induced signaling pathways by exposure to IGF-I and/or chemotherapy and using pharmacologic and molecular techniques to confirm these interactions.

Our interesting observation that IGF-I treatment markedly enhances JNK activity in a PI 3-kinase dependent fashion in MCF-7 cells has recently been a major focus in my lab. We have shown that IGF-I activation of JNK is stronger than that induced by stress treatments such as Taxol or Taxotere chemotherapeutic agents. When cells are co-treated with IGF-I and chemotherapy, JNK activity is further enhanced (in contrast to our prediction). Thus, we have been very interested in the biological consequence of JNK activation by growth factors. In the past few months we have made significant progress. We have submitted a manuscript to *Oncogene* that describes that overexpression of wildtype JNK inhibits anchorage independent growth of MCF-7 cells and IGF-I stimulated anchorage independent growth. We believe that IGF-I activation of JNK is resulting in a negative feedback loop. Other investigators have shown in diabetic models that stress treatments such as TNF-α (tumor necrosis factor) enhances JNK activity. Activated JNK then phosphorylates IRS-1 (insulin receptor substrate) on Serine 307. Phosphorylation of this residue inhibits IRS-1 association with insulin and IGF-I receptors, resulting in decreased growth factor mediated signaling. We find that that IGF-I activated JNK is working similarly to inhibit breast cancer growth. Our data suggest that JNK may be an excellent therapeutic target; its activity enhances chemotherapy induced apoptosis and inhibits growth factor mediated proliferation or survival.

In November of 2001, we received an $50,000 Avon seed grant to continue our studies of IGF-I activated JNK. The aims of this grant are to identifying upstream kinases that mediate IGF-I activation of JNK and to inhibit JNK isoforms to determine if IGF-I activates different JNK isoforms than stress treatments. We are approaching this question through a collaboration with ISIS Pharmaceutical company who have provided us with JNK antisense oligonucleotides. We believe we have optimized the treatment conditions in our model and are poised to prepare a manuscript within the next two to three months. We and others believe that JNK isoform specificity may contribute significantly to treatment dependent effects of JNK.

Task 3 is to characterize if IGF-I and chemotherapy interactions in breast cell lines are p53 dependent and to compare this activity in an ER positive cell line, and ER negative cell line, and an immortalized, noncancerous breast epithelial cell line.

In the DoD grant, I proposed to study both mutant p53 and wildtype p53 expressing breast cancer cell lines. We have done this to some extent but have also had difficulty in deciphering general differences in cell line sensitivity versus differences in mutant versus wildtype p53 function. Thus, we have obtained a p53 null breast cancer cell line, 21PT. We are currently transfecting this cell line with wildtype 53 so that we can better compare p53 specific responses and the mechanism for Akt inhibition of p53 mediated apoptosis.

Task 4 is to study IGF-I treatment effects on xenograft breast cancer response to chemotherapy drugs.

We have recently submitted both an Idea and RO1 application to use established transgenic mouse models and JNK knockout mice to better understand the interactions of JNK with growth factor mediated pathways and the efficacy of chemotherapy drugs. Based on the literature and our own preliminary data, we hypothesize that loss of JNK activity will *enhance* IGF-I and polyoma virus middle T-antigen (PyV MT) mediated breast tumorigenesis, possibly via PI 3-kinase activation. In contrast, loss of JNK activity in mutant (mt) p53 expressing cells will *inhibit* mammary tumorigenesis. Once breast tumors have developed, we then plan to determine if JNK expression is important for efficacy of anti-cancer drugs. We predict that loss of JNK will enhance tumor response to tamoxifen while inhibiting Taxol response. We will determine if these effects are JNK gene dependent by using both JNK 1 and JNK 2 knockout mice. If either of these applications are funded, these aims will supplant the current Task 4 of my CDA award. I believe that use of the transgenic and knockout mice will address the same question but provide a much more mechanistic information to the initial approach described.

Training accomplishments

This past year has been marked by better refinement of our research tools. Dr. Horwitz (my mentor) has been instrumental in earning the Avon breast cancer foundation award. Seed funds from this award provided support for many of the JNK research currently taking place in my laboratory. This Foundation award has brought breast cancer researchers from the area in closer contact and more collaborative. In regards to my career development, I have also served as a grant reviewer for both DoD and NIH. This has given me a great opportunity to stay abreast of breast cancer research and better reflect upon my own research program's focus and progress.

Key Research accomplishments:

1. Characterization of IGF-I activation of JNK inhibits IGF-I responses in breast cancer cells (see attached appendix)
2. Collaboration with ISIS Pharmaceutical company to study JNK isoform specificity of function.
3. IGF-I survival responses in DNA damaged breast cancer cells are caspase 9 independent but caspase 7 sensitive.

Reportable Outcomes:

1. Avon seed grant award
2. Submission of grant applications to DoD and NIH entitled, "The role of JNK in mammary tumorigenesis and response to treatment."
3. Submission of a manuscript to *Oncogene* entitled, "An inhibitory function for JNK in the regulation of IGF-I signaling in breast cancer."
3. Abstract acceptance to Era of Hope meeting in September 2002, entitled, "Molecular targets for IGF-I protection of irradiated breast cancer cells."

Conclusions: We have had a very productive year in characterizing the IGF-I induced pathways and the mechanism(s) of their effects on cellular outcomes. Our findings that JNK activation inhibits IGF-I responses and that JNK activity mediates chemotherapy induced cell death provides us with a very promising target for therapeutic interventions. We first would like to confirm and extend our studies using animal models, thus we have grant applications pending to do such studies. The other aspect of our research which focusing on Akt mediated survival suggest that Akt is not working through caspase 9, as published in other models. Currently we are narrowing in on the potential new targets for Akt inhibition of apoptotic pathways. We hope to submit this work for publication within the next two months. Since Akt has been frequently reported to convey protective responses in cancer cells, identifying its target(s) in DNA damaged cells may provide us with ways of offsetting resistance to current therapeutic modalities.

An inhibitory function for JNK in the regulation of IGF-I signaling in breast cancer

Cindy L. Mamay[*], Amy M. Mingo-Sion[‡], Doug M. Wolf[¥‡], Marion D. Molina[‡], Carla L. Van Den Berg[§‡]

[‡] School of Pharmacy, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Campus Box C238, Denver, CO 80262

*University of California, Davis, Department of Biological Chemistry, Davis, CA 95616-8655
[¥] Department of Obstetrics and Gynecology, School of Medicine, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Campus Box B198, Denver, CO 80262

Keywords: IGF-I, JNK, IRS-1, breast cancer, apoptosis

[§] To whom correspondence should be addressed: Carla Van Den Berg, School of Pharmacy, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Campus Box C238, Denver, CO 80262. Tel.: (303) 315-5948; Fax: (303) 315-4630; E-mail: carla.vandenberg@UCHSC.edu

Abstract

IGF-IR (insulin-like growth factor-I receptor) is frequently overexpressed in a variety of cancer types. Since many breast tumors and cancer cell lines overexpress IGF-IR, we tested IGF-I effects on chemotherapy treated breast cancer cells. IGF-I protects from chemotherapy-induced apoptosis, suggesting that overlapping signaling pathways modulate IGF-I and chemotherapy treatment outcomes. Taxol and other chemotherapy drugs induce c-Jun N-terminal kinase (JNK), a kinase that conveys cellular stress and death signals. Notably, in this paper we show that IGF-I alone induces a potent JNK response and this activity is reversed by inhibition of phosphatidylinositol 3-kinase (PI 3-kinase) with LY294002 in MCF-7 but not T47D cells. Co-treatment of cells with chemotherapy and IGF-I leads to additive JNK responses. Using cells overexpressing Akt, we confirm that IGF-I mediated survival is Akt dependent. In contrast, overexpression of JNK significantly enhances Taxol induced apoptosis and inhibits IGF-I survival effects. Further, JNK attenuates anchorage independent growth of MCF-7 cells. The inhibitory effect of JNK appears to be mediated by serine phosphorylation of IRS-1 (insulin receptor substrate) since both Taxol and IGF-I treatment enhanced Ser^{312} IRS-1 phosphorylation, while LY294002 blocked IGF-I mediated phosphorylation. Taken together, these data provide a mechanism whereby stress or growth factors activate JNK to reduce proliferation and/or survival in breast cancer cells.

Introduction

The importance of IGF-IR action in breast cancer has been clearly demonstrated. Its expression has been observed in 87% of breast cancer specimens (Peyrat et al., 1990) and overexpressed IGF-IR in human tumor specimens is functional, having increased kinase activity compared to normal mammary tissue (Resnik et al., 1998). Furthermore, Cullen and colleagues (Cullen et al., 1990; Singer et al., 1995) have shown that breast cancer evolution is associated with the induction of IGF-II secretion, another IGF-IR ligand, from stromal cells neighboring malignant breast tissue. Butler and colleagues (Butler et al., 1998) have shown that systemically administered IGF-I stimulates tumor growth in mice in a dose-dependent fashion using NIH 3T3 cells overexpressing IGF-IR. Inhibition of IGF-IR activity by overexpression of a dominant negative form of IGF-IR in MDA-MB-231 and MDA-MB-435 breast cancer cell lines inhibits the adhesion, invasion and metastasis of these cells, as well as enhances their sensitivity to Taxol-induced cell death (Dunn et al., 1998). Some of these IGF-IR mediated tumorigenic properties may be attributed to its central role in the regulation of proteins important for either matrix attachment or cell survival.

Activated IGF-IR imparts both survival and proliferative effects in various experimental models including neuronal and cancer cells. After IGF-IR autophosphorylation, IGF-IR complexes with and tyrosine phosphorylates IRS docking proteins which then activate second messengers, such as MAPK (mitogen activated protein kinase; also known as ERK (extracellular signal-related kinase)) and PI 3-kinase. Recently, many significant observations have been published regarding the role of serine/threonine kinases that phosphorylate IRS-1. Interestingly, serine phosphorylation of IRS-1 by downstream kinases can regulate IRS-1 tyrosine phosphorylation status and its ability to serve as a substrate to IR (insulin receptor) or IGF-IR.

Based on IRS-1's sequence, there are 35 potential serine/threonine phosphorylation sites. Some of these sites, when phosphorylated, result in either sustained or abbreviated IRS tyrosine phosphorylation, modifying downstream responses in a positive or negative fashion. Activation of pathways involving PI 3-kinase, Akt, GSK3 (glycogen synthase kinase-3), MAPK, and JNK (amongst others) are known to serine phosphorylate various IRS-1 sites and presumably the duration of IGF-I or insulin signaling . Further, IRS-1 signaling may be mediated by proteasome degradation subsequent to ligand binding; this activity is also mediated through PI 3-kinase but not MAPK (Lee et al., 2000).

Soon after PI 3-kinase activity is enhanced by binding to IRS-1, activation of downstream kinases Akt and/or p70 S6 kinase occurs. PI 3-kinase activation of Akt conveys many growth factor dependent survival effects (Kennedy et al., 1997). Enhanced Akt activity results in inhibition of many cell death proteins through phosphorylation of BAD (BCL-X_L/BCL-2 associated death promoter) (Datta et al., 1997), the protease caspase-9 (Cardone et al., 1998), and the forkhead transcription factor family (Brunet et al., 1999).

In addition to inducing PI 3-kinase dependent responses, IGF-I also strongly activates MAPK in MCF-7 and T47D breast cancer cells. MAPK inhibition by the pharmacologic agent PD0908059 reduces proliferation of MCF-7 and T47D cells, thus implicating a role for MAPK in IGF-mediated proliferation (Hermanto et al., 2000). Another MAPK family protein, that is also activated by IGF-I is JNK (Monno et al., 2000); however, the function of this activation is poorly understood. In fact, others have shown that IGF-I pre-treatment inhibits JNK activity induced by TNFα (tumor necrosis factor α), and anisomycin in 293 cells (Okubo et al., 1998). Since JNK is activated by diverse cell stimuli, including stress, growth factors (i.e. EGF (epidermal growth factor)), and cytokines (i.e. TNFα and Interleukin-1) (Rosette & Karin, 1996;

Sluss et al., 1994; Whitmarsh et al., 1995) JNK mediated effects can be pro-apoptotic, proliferative, or anti- proliferative.

JNK is best characterized by its sensitivity to stress treatments like anisomycin, irradiation, and TNFα. Many chemotherapy drugs also induce JNK as a result of DNA damage or microtubule interference. In particular, microtubule-interfering agents (MIAs), such as paclitaxel (Taxol) and docetaxel (Taxotere), activate JNK through pathways involving Ras and ASK1 apoptosis signal-regulating kinase-1 (Chen et al., 1996; Wang et al., 1998), indicating that activation of JNK is needed to initiate the microtubular disarray caused by MIAs. Thus, there is considerable evidence to support the role of JNK in regulating apoptosis following cellular stress. When JNK activity is enhanced by chemotherapeutic agents and radiation, it signals cells to undergo programmed cell death (Chen et al., 1996; Wang et al., 1998).

In this study we investigated mechanism(s) of IGF-I survival responses in chemotherapy treated breast cancer cells. We focused on JNK activation by the MIA, Taxol, and sought to determine if this activation might be altered by IGF-I co-treatment in breast cancer cells. We initially hypothesized that IGF-IR chemoprotective effects in our model result from stimulation of PI 3-kinase and its downstream effector, Akt. Here, we confirm that Akt has a significant role in IGF-I mediated survival; however, we also show that IGF-I treatment alone potently activates JNK in breast cancer cell lines. In MCF-7 cells, but not in T47D cells, this action is significantly reduced by PI 3-kinase inhibition. Co-treatment of breast cancer cells with Taxol and IGF-I results in an additive increase in JNK activity. The resulting cellular outcome of JNK overexpression is attenuation of IGF-I responses. These inhibitory effects of JNK on IGF-I responses appear to result from phosphorylation of Ser^{312} (corresponding to rat Ser^{307}) on IRS-1.

Altogether, these results suggest that IGF-I may downregulate its own survival effects via activation of JNK, thus abrogating its ability to promote cell survival or proliferation.

Materials and Methods

Cell Culture and Treatments- MCF-7 cells were provided by C. Kent Osborne (San Antonio, TX) and T47D cells were obtained from the University of Colorado Cancer Center, Tissue Culture Core (Denver, CO). Both cell lines were maintained in full media (IMEM (Improved Minimal Essential Medium)) with phenol red (Mediatech, Herndon, VA)) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA), antibiotics, glutamine, and insulin. In each experiment, cells were plated in full media and cultured overnight at 37°C and 5% CO_2. The following day, cells were washed twice with warm PBS (phosphate buffered saline, Biofluids, Rockville, MD) and then cultured overnight in SFM (serum free media). The next day, cells were treated with IGF-I (Bioreclamation, Hicksville, NY and the National Hormone and Pituitary Program obtained from NHPP, NIDDK & Dr. A.F. Parlow), Taxol (paclitaxel (Mead Johnson, Princeton, NJ)), or Taxotere (docetaxel (Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA)) as described in figure legends. Pretreatments with either 100 nM wortmannin or 50 to 100 μM LY294002 as indicated (Calbiochem and Alexis Biochemicals, respectively, San Diego, CA) were performed 40 minutes prior to stimulation with IGF-I. All protein concentrations from cell extracts were determined using a Bio-Rad D/C protein assay kit (Bio-Rad, Hercules, CA).

Transfection experiments- Hemagglutinin (HA) tagged Akt and Akt (K179A, kinase dead) vectors were graciously provided by M.E. Greenberg (Datta et al., 1997). The myr-HA-Akt

pcDNA3 construct was a gift from J.R. Testa (Mitsuuchi et al., 2000). JNK and JNK (T183A/Y185F, kinase dead) pcDNA3 constructs were obtained from Dr. C. Franklin, University of Colorado Health Sciences Center. Generation of both wild-type (Wt) and mutant (Mt) Akt and JNK stable transfectants was performed by plating 2 x 10^6 of MCF-7 cells per 10 cm dish. The following day, cells were transfected with 10 μg of DNA by lipofection (Lipofectamine™, Gibco BRL, Grand Island, NY). Control cells were transfected with empty vector. Twenty-four to 48 hours later cells were selected with 800 μg/ml of G418. Individual resistant colonies were isolated and expanded. Detection of clones overexpressing either Akt and JNK Wt or Mt genes was performed by Western blot analysis using HA-probe (F-7) primary antibody (sc-7392, Santa Cruz Biotechnology, Santa Cruz, CA). For transient transfections, 1.8 x 10^6 MCF-7 cells were plated in 60mm dishes. The next day cells were transfected with using Lipofectamine™ and Plus™ reagent according to product(s) instructions (Gibco BRL, Grand Island, NY). Cells were then placed in serum free media overnight prior to treatment.

JNK assay and Western blot analysis- As a positive control for JNK activation, cells were plated and serum starved as described previously. UV irradiation was performed by exposing cells to UVC (ultraviolet-C, 50 J/m^2) in a Strategene UV linker 1800 (Stratagene, La Jolla, CA). Cells were then incubated at 37^0C in 5% CO_2 for 50 minutes before preparing cell lysates. Other cells were plated and serum-starved and then exposed to either IGF-I and/or chemotherapy as indicated. Tissue culture dishes were washed twice with ice-cold PBS. Cells were harvested and *in vitro* kinase assays were performed as described previously (Hibi et al., 1993) using cell lysate volumes corresponding to 400 to 600 μg of total protein. The products were then resolved by 10% SDS-PAGE (polyacrylamide gel electrophoresis). The gel was dried and subjected to

radiography. Additionally, phosphorylated c-jun product incorporating ^{32}P was quantitated by PhosphorImager analysis.

To assure that kinase reactions in each experiment contained approximately equal amounts of JNK protein per sample, 10 µl of supernatant from each kinase reaction were run on a 10% SDS-PAGE. Proteins were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) and JNK protein quantity was verified by immunoblotting with JNK1 antibody (FL, Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence (Amersham, Piscataway, NJ).

Akt substrate construct, affinity purification, immobilized and soluble GST thrombin-Akt substrate- The peptide RPRAATF, corresponding to the sequence containing the threonine phosphorylation site of GSK-3, was fused in frame with GST in pGEX-4T-1 by first hybridizing the oligonucleotides (5'-AATTGCGTCCGCGTGCTGCCACCTTCG-3') and (5'-AATTCGAAGGTGGCAGCACGCGGACGC-3') to produce a double stranded DNA with EcoRI sticky ends. This DNA fragment was then cloned into the EcoRI site of pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ), regenerating only one EcoRI site. A construct with the insert in the correct orientation was identified by restriction analysis and sequencing (University of Colorado Cancer Center DNA Sequencing Core Service). Affinity purification of the GST thrombin-Akt substrate fusion protein was performed as described by Smith et al. (Smith & Johnson, 1988).

Akt assay- After IGF-I treatment, tissue culture dishes were washed twice with ice-cold PBS. Cells were harvested in lysis buffer and kinase assays performed, as previously described (Franke et al., 1995), using volumes of pre-cleared lysate containing 400 µg of total protein for

each sample. Reactions were terminated with 15 µl of 4X sample buffer. The products were then resolved by 10% SDS-PAGE. The gel was dried and subjected to radiography. Additionally, phosphorylated product incorporating ^{32}P was quantitated by PhosphorImager analysis. Immunoprecipitation or Western blot analysis of Akt1 was performed using anti-Akt1 antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA).

Apoptosis Assays- 75,000 MCF-7 cells were plated in each 1.7 cm^2 chamber of Biocoat CultureSlides™ (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Cells were cultured overnight as described above. The following day, cells were washed twice with PBS and then exposed to SFM control, IGF-I 50 ng/ml alone, Taxol 0.02 µM alone, or Taxol plus IGF-I as described in figure legends. Forty-eight hours after exposure to treatment cells were fixed in 3% formaldehyde in PBS for five minutes, washed three times with PBS, and nuclei were stained with a solution containing 15 µM Hoechst 33258 in PBS for 10 minutes. A minimum of 200 cells were counted per treatment and the nuclei morphology determined as viable or apoptotic using a fluorescence microscope (Zeiss, Oberkochen, Germany).

PARP (poly(ADP) ribose polymerase) cleavage was measured in cells transiently transfected (see above) with myr-HA-Akt-pcDNA3 or empty pcDNA3 vector. Additionally, PARP experiments using overexpressed JNK were from the stably transfected MCF-7 cells. Cells were treated as described above and lysed using EB buffer (20 mM Tris-HCl, pH 7.6, 0.25 M NaCl, 3 mM EDTA, 0.5% IGEPAL CA-630 with freshly added 1mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride), 20 µg/ml aprotinin, 5 µg/ml leupeptin, and 2 mM Na orthovanadate). Seventy micrograms of protein were then resolved by 10% SDS-PAGE.

Proteins were transferred to nitrocellulose and Western blots performed using anti- PARP antibody (H250, sc-7150, Santa Cruz Biotechnology, Santa Cruz, CA).

IRS-1 Immunoprecipitation and Western Blot Analysis: Non-transfected and transfected cells were treated with IGF (50 ng/ml) and/or LY294006 (50 μM) according to figure legends. Cells were transfected with either empty vector or pEBG-JNK1 (a gift from Dr. Kyriakis, Harvard University) according to method described above. Following culture and/or transfection, cells were serum starved overnight prior to treatment. Following treatment, cell lysates containing 300 μg of protein were incubated with 2.4 μg of anti-IRS-1 antibody (Upstate Biotechnology, Lake Placid, NY) and 30 mcg of rProtein G Agarose beads (Gibco, Gaithersburg, MD) overnight at 4°C. Beads were washed three times in lysis buffer and re-suspended in sample buffer. Samples were loaded on a 7% SDS-page acrylamide gel, then transferred to nitrocellulose. Membranes were blocked in 5% milk in TBST, then incubated with either anti-phospho- IRS-1 (Ser^{307}) (Upstate Biotechnology, Lake Placid, NY) or an anti-phosphotyrosine antibody (RC20, BD Transduction, San Diego, CA). All Western blots were analyzed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Data Analysis: Apoptosis assays were analyzed by multiple logistic regression performed using SAS/PROC GENMOD, version 6.12 (SAS Institute, Carey, NC). Tests were performed for overall effect of IGF or the transfected gene on Taxol induced apoptosis, as well as for significant interaction effects between IGF and the transfected gene. As multiple comparisons were required to address all questions of interest, results were not considered statistically significant unless the contrast reached $p < 0.005$.

Results

MIA treatment activates JNK in breast cancer cells

The various biological outcomes of IGF-I treatment of breast cancer cells have been well described. However, less is known about the signaling pathways that allow IGF-I to convey its cytoprotective effects in chemotherapy treated cancer cells. We initially set out to measure JNK response to chemotherapy in our model, and then to determine if IGF-I treatment might suppress stress-related signaling as a possible mechanism for its inhibition of programmed cell death. JNK activation in response to chemotherapy treatment has been suggested to correspond to cell sensitivity to chemotherapy (Potapova et al., 1995). Therefore, we tested if the chemotherapeutic agents, doxorubicin, Taxol, and Taxotere, induce JNK activity in MCF-7 breast cancer cells. Treated cells were harvested at various time points to determine the peak time of activation. Figure 1 illustrates that Taxol and Taxotere treatment of cells induced JNK activity approximately 3-fold, while JNK activation by doxorubicin is somewhat less than 3-fold. A bimodal pattern of JNK activation was observed with Taxol treatment with early activation occurring at two hours of exposure and maximal activation observed at eight hours. Taxotere treatment led to similar levels of JNK induction as Taxol ($\geq$ 3-fold over control), however only one peak activation time occurred at two hours of exposure.

IGF induces JNK via PI 3-kinase in MCF-7 cells

In order to study IGF-I's ability to regulate JNK in breast cancer cells, we measured the effect of IGF-I treatment alone on JNK activity. Initially, we predicted that IGF-I treatment alone would either, 1) suppress JNK signaling, since many treatments that stimulate JNK result in cell death and IGF-I opposes this response, or 2) have minimal effect on JNK, as other investigators have shown that tyrosine kinase receptor activation leads to low levels of JNK

activity, generally peaking within 30 minutes of exposure (Alblas et al., 1998; Fanger et al., 1997; Goedert et al., 1997; Kyriakas et al., 1994; Westwick et al., 1994). Figure 2 shows that IGF-I potently stimulates JNK in both MCF-7 and T47D breast cancer cells. Maximal JNK activity was observed in MCF-7 cells after two hours of exposure, when approximately a 9-fold level of induction was typically observed in multiple experiments with MCF-7 cells, this JNK induction was notably more robust activation than what we observed with the chemotherapy agents. In T47D cells (Figure 2), JNK activation peaked at 30 minutes and was somewhat less than that observed in MCF-7 cells.

Since PI 3-kinase is a known second messenger for IGF-IR signaling, we sought to then determine if IGF activation of JNK is PI 3-kinase dependent. Addition of the PI 3-kinase inhibitors, wortmannin and LY294002, to IGF-I treated MCF-7 cells significantly reduced IGF-I dependent activation of JNK (Figure 3). Although, IGF-I induced JNK activity in both breast cancer cell lines, use of both PI 3-kinase inhibitors showed that IGF-I activation of JNK was PI 3-kinase dependent in MCF-7 cells but not in T47D cells (Figure 3). These data support the hypothesis that PI 3-kinase lies upstream of JNK in MCF-7 cells treated with IGF-I and suggests that IGF-I activation of JNK through PI 3-kinase may influence IGF-I and/or Akt survival responses.

Combined IGF-I and MIA treatment results in an additive JNK response

We then assessed endogenous JNK response to co-treatment using IGF-I and chemotherapy to determine if these two treatments might counteract one another's signaling through JNK. UV treatment was also studied in co-treatment of cells to ascertain if IGF-I effects on JNK agonists could be extended to other stress treatments. MCF-7 cells were pretreated with either Taxol (for five or seven hours) or Taxotere (for 1 hour) then IGF-I was added for the last

hour of exposure, in order to harvest cells at the approximate times of maximal activity for each treatment. Figure 4A and B show JNK phosphorylation of c-Jun substrate under the conditions described above. In contrast to a previous report (Okubo et al., 1998) and our predictions, IGF-I co-treatment of cells did not interfere with JNK activation by other JNK activating agents. In fact, JNK induction was higher with co-treatment when IGF-I was added after stress treatment (Figure 4) or simultaneously (data not shown). The greatest JNK activity was observed when cells were treated for the duration of time seen for maximal activity with each treatment alone. These conclusions can be extended to other stress treatments that induce JNK activity like Taxotere and UV (Figure 4C and D). Western blots assessing total JNK protein in kinase reactions confirm that changes in JNK activity are not a result of changes in the amount of JNK protein in the various samples. Because of the large increase in JNK activity induced by UV, significant increases with IGF-I co-treatment were more difficult to observe. Since co-treatment with IGF-I and chemotherapy led to an increase in JNK activity, we sought to determine if this activation is reduced by PI 3-kinase inhibition. Wortmannin inhibited JNK activity in IGF-I treated samples (Figure 4A and B). These data show that both stress treatment and IGF-I treatment enhance JNK activity and indicate that IGF-I survival responses may be mediated in a complex manner involving both JNK and Akt activation. JNK activation by both stress and growth factor treatment may also indicate complex signaling through different isoforms, resulting in different biological responses (Chen et al., 2001; Potapova et al., 2000; She et al., 2002). Next, we set out to characterize the roles of IGF-I activated JNK and Akt on cellular outcome(s).

Akt, but not JNK, enhances IGF-I cytoprotection

First, we needed to confirm that Akt is activated by IGF-I treatment in MCF-7 cells. We developed a kinase specific substrate using the Akt consensus binding sequence of GSK (a well-characterized Akt substrate) to investigate the involvement of downstream Akt activity in PI 3-kinase dependent survival in our model. *In vitro* kinase assays confirmed that IGF-I treatment of MCF-7 cells induced Akt maximally at 10 minutes of exposure (Figure 5A) and that pre-treatment with the PI 3-kinase inhibitors, LY294002 (Figure 5B) or wortmannin (data not shown), reduced IGF-I induction of Akt. Increased Akt activity was observed both when Akt was isolated by immunoprecipitation (data not shown), and when Akt containing cell lysates were exposed to an immobilized Akt specific substrate, GST-GSK, following IGF-I treatment. These results support that PI 3-kinase and Akt may elicit IGF-I survival effects in MCF-7 cells. In T47D cells, Akt is phosphorylated even in the absence of growth factor indicating that PI 3-kinase activity may be constitutive in that cell line (data not shown).

In order to more clearly decipher the potential role of either JNK or Akt in IGF-I cytoprotection, MCF-7 cells were stably transfected with either Wt Akt, Wt JNK, Mt Akt (K179A, kinase dead), or Mt JNK (T183A/Y185F, kinase dead) hemagglutinin tagged constructs. Stable transfectants were plated in chamber slides, serum starved overnight, and treated cells were exposed to Taxol or IGF-I treatment alone or in combination. Figure 6A shows that IGF-I significantly reduced apoptosis induced by Taxol, (test for overall effect, $p<0.0001$). IGF-I reduced cell death in parental and Mt Akt transfected cells by 59% and 53%, respectively, compared to Taxol treatment alone. Cells overexpressing Wt Akt experienced a 34% reduction in apoptosis with IGF-I treatment, ($p=0.056$). The lack of a significant effect of IGF-I on Wt Akt expressing cells appears to be due to the dramatic effect that overexpression of Wt Akt has on Taxol's ability to induce apoptosis in these cells. Wt Akt overexpressing cells

underwent 70% less apoptosis than parental MCF-7s treated with Taxol alone (13% vs 44%, $p < 0.0001$). We did not observe a significant difference between Mt Akt transfectants and parental cells with respect to IGF-I protection from Taxol induced apoptosis. These latter results suggest either that another IGF-I dependent pathway may counteract Mt Akt effects or that Mt Akt overexpression was not able to squelch endogenous Akt signaling in response to IGF-I.

Cells transfected with Wt or Mt JNK were treated in a similar fashion as described above (Figure 6B). Overall, parental control groups were very similar to those in Akt experiments; in JNK experiments IGF-I reduced Taxol induced apoptosis in parental cells by 60% versus 59% in Akt experiments (Figure 6A and B). Comparison of all treatment groups of Wt JNK transfectants to parental cells demonstrates that overexpression of Wt JNK results in a significant increase in apoptosis (test for overall effect, $p=0.0001$). Figure 6B also shows that, similar to the effects of Wt Akt, transfection of cells with Mt JNK leads to a dramatic decrease in cell sensitivity to Taxol compared to parental cells, 19% versus 47% apoptosis ($p=0.0009$). This lends further support to the hypothesis that JNK activity is transducing a cell death signal under cellular stress. Similar to the Wt Akt transfected cells, we did not observe significant increases in protection with Mt JNK overexpression when cells were co-treated with IGF-I.

Since the Hoechst apoptosis assay may be somewhat subjective, significant results were confirmed using PARP cleavage analysis using separate stable transfectant lines, shown in Figure 7. Using this assay, parental MCF-7 cells underwent apoptosis, as indicated by the appearance of a PARP cleavage fragment at 85 kD, when treated with Taxol. Again, IGF-I protected from Taxol-induced PARP cleavage at Taxol concentrations up to 0.04 μM (Figure 7A). To clarify the role of Akt in our model, we inhibited PI 3-kinase using LY294002, and we also determined the contribution of activated Akt in a fashion that eliminates other IGF-I and PI

3-kinase dependent pathways by using the myristylated form of Akt (myr-Akt) in transient transfections. Pre-incubation of MCF-7 cells with LY294002 enhanced Taxol mediated PARP cleavage and inhibited IGF-I protection of cells (Figure 7B), while overexpression of myr-Akt blocked Taxol-induced apoptosis (Figure 7C), confirming that Akt mediates cell survival in Taxol treated cells. However, data including the LY294002 compound must be interpreted with caution since this agent also inhibits JNK in our model. In contrast to these Akt data, overexpression of Wt JNK alone enhanced Taxol-induced PARP cleavage (Figure 7D). Further, using the Wt JNK transfectants we were still unable to demonstrate that JNK activation by IGF-I had any effect on cell survival. These observations may be explained either by the lack of a role for JNK in conveying an IGF-I survival responses or by a dominant effect of Taxol induced JNK. The last possibility, which we were unable to ascertain in these studies, is the presence of JNK isoform responses that may be treatment dependent.

JNK inhibits MCF-7 anchorage independent growth

We have previously established that IGF-I treatment of MCF-7 cells enhances anchorage independent growth (AIG) (Van Den Berg et al., 1997). In order to determine if IGF-I induction of JNK results in a change in IGF-I mediated responses in the absence of a strong stress treatment, we assessed the effect of JNK overexpression on IGF-I enhanced colony formation (Figure 8). Stable transfectants of Wt and Mt JNK were compared to parental MCF-7 cells. Wt JNK overexpression alone strongly inhibited MCF-7 colony formation (comparing parental MCF-7 cells to Wt JNK transfectants), and it also blocked IGF-I enhancement of colony formation (comparing Wt JNK transfectants to Wt JNK transfectants + IGF-I), suggesting that JNK activation by IGF-I antagonizes IGF-I tumorigenic effects. Both Mt JNK transfectant samples, cultured in 5% charcoal stripped serum (CSS) and 5% CSS + IGF-I treatment, formed

increased numbers of colonies that were similar to the parental MCF-7, IGF-I treated controls. These data support the conclusion that IGF-I induction of JNK is inhibiting IGF-I proliferative or survival effects in MCF-7 cells.

Phosphorylation of IRS-1 human Ser^{312} is PI 3-kinase dependent and increased in Wt JNK transfectants.

Given our evidence that JNK inhibits IGF-I responses and that of other investigators showing that JNK can bind to IRS-1 and serine phosphorylate human Ser^{312}, we decided to determine if this response could be a potential mechanism for the observed inhibitory effect of JNK by IGF-I treatment in our cells. Parental MCF-7 cells were pre-treated with LY294002 in some instances prior to treating the cells with IGF-I or Taxol. Total IRS-1 was immunoprecipitated and isolated protein was analyzed using a rat IRS-1 antibody Ser^{307} (corresponding to human Ser^{312}) for Western blot analysis. Figure 9A shows that IGF-I treatment of parental MCF-7 cells enhances phosphorylation of Ser^{312} at times where peak JNK activation by IGF-I was observed in these cells. Further, this activity was blocked by inhibiting PI 3-kinase with LY294002. Taxol treatment also increased Ser^{312} phosphorylation (Figure 9B), indicating that JNK may be mediating phosphorylation of this serine site. In Figure 9C, cells were transfected using either empty vector or a GST-Wt JNK containing plasmid. After serum starvation, transfected cells were treated with IGF-I and LY294002 as described above. IRS-1 was immunoprecipitated from cleared cell lysates using a total IRS-1 antibody. Western blot analysis was performed using the Ser^{307} IRS-1 antibody. Again, IGF-I treatment of cells enhances Ser^{312} phosphorylation and LY294002 pre-treatment inhibits IGF-I dependent serine phosphorylation. In these experiments, an increase in IGF-I mediated serine phosphorylation was observed in the Wt JNK transfectants compared to mocked transfected cells. Further, pre-

treatment with LY294002 inhibited serine phosphorylation in both mock and Wt JNK transfected cells, supporting the conclusion that IGF-I mediated phosphorylation of Ser^{312} is PI 3-kinase dependent. Further, we assessed the potential role of JNK activity on tyrosine phosphorylation of IRS-1. Immunoprecipitates of total IRS-1 were analyzed for tyrosine phosphorylation by Western blot analysis using an anti-phosphotyrosine antibody. Tyrosine phosphorylation of IRS-1 was robust after one hour and somewhat less at two hours of IGF-I treatment. Co-treatment with the LY294002 did not appear to inhibit tyrosine phosphorylation but these samples of IRS-1 migrated more rapidly, possibly indicating less serine phosphorylation. Interpretation of IRS-1 tyrosine phosphorylation data using the LY294002 inhibitor is complex, given that it also inhibits Akt. Akt phosphorylates IRS-1 at sites not including Ser^{312}, however, this serine phosphorylation activity is thought to prevent tyrosine de-phosphorylation of IRS-1 (Paz et al., 1999). Finally, Wt JNK transfectant cells had slightly less tyrosine phosphorylation compared to MCF-7 cells. With these considerations in mind, our data support the conclusion that IGF-I activated JNK may result in serine phosphorylation of IRS-1, but it is unclear if this action results in an inhibitory tyrosine phosphorylation effect on IRS-1 and its ability to mediate IGF-I signaling in breast cancer cells.

Discussion

In this paper we demonstrate that IGF-I has potent effects on JNK and Akt activity. IGF-I activation of both PI 3-kinase and Akt supports the hypothesis that this pathway is essential for IGF-I mediated chemoprotection. These data, along with apoptosis experiments using Wt and Mt Akt transfectants, further confirm our previous results showing that pharmacologic inhibition of PI 3-kinase and its downstream kinases enhanced both doxorubicin and Taxol induced

apoptosis (Gooch et al.,). Additionally, use of a myr-Akt construct confirmed that enhanced Akt activity has a strong cell survival effect.

The large degree of JNK activation by IGF-I was somewhat surprising since activation of other tyrosine kinase receptors does not typically induce JNK to the same level or time to maximal stimulation as we observed (Alblas et al., 1998; Fanger et al., 1997; Goedert et al., 1997; Kyriakas et al., 1994; Westwick et al., 1994). For example, other investigators (Desbois-Mouthon et al., 1998; Miller et al., 1996) have reported that insulin treatment of CHO or Rat 1 HIR fibroblasts activates JNK within 10 to 15 minutes of exposure. This activity results in an increase in AP-1 DNA binding and possibly cell proliferation (Miller et al., 1996). Given that IGF-I induction of JNK is notably higher than what has been reported with other growth factors and since other growth factors induce proliferation through JNK (Bost et al., 1997), we were initially perplexed by our results showing a lack of IGF-I proliferative (data not shown) or survival responses through JNK.

Activated PI 3-kinase has previously been shown to be upstream of JNK after exposure to other growth factors like PDGF (platelet-derived growth factor), and in some cases EGF, but not after exposure to UV irradiation or osmotic shock (Logan et al., 1997; Lopez-Ilasaca et al., 1997). Other investigators report that despite IGF-I's limited ability to induce JNK in embryonic kidney 293 cells, pre-treatment with IGF-I suppresses JNK stimulation by TNF-α and anisomycin (Okubo et al., 1998). These investigators stressed the necessity of IGF-I pre-treatment in order to observe inhibition of JNK in response to cellular stress but we did not study the effects of IGF-I pre-treatment in our model. Also, these investigators did not study JNK response to IGF-I treatment alone beyond one hour of exposure. In our model, we observed maximal activity after two hours of IGF-I exposure. Given these mixed results reported by

others, we initially anticipated that if IGF-I had any measurable effect on JNK it might be to abrogate chemotherapy induction of JNK. It is now clear that although chemotherapy drugs activate JNK, IGF-I induced JNK to a far greater extent in our breast cancer model. Co-treatment with chemotherapy and IGF-I further enhanced JNK activity, despite their opposing effects on cell survival.

The biological effect of overexpressed JNK suggests that even though it was induced both by Taxol and IGF-I, JNK's primary effect appears to be pro-apoptotic rather than protective. Moreso, IGF-I lost its cytoprotective effect in cells overexpressing JNK, where the stoichiometry of JNK versus Akt protein levels may have allowed JNK induced apoptotic response to overcome some of the IGF-I mediated Akt effects in these cells. Further, we show that JNK activity inhibited IGF-I responses, in the absence of stress, by reducing colony formation in the AIG assay and inhibiting the IGF-I mediated increase in colony growth. Thus, we turned our attention to potential mechanisms of inhibition of IGF-IR signaling.

Much attention has recently been drawn to IRS phosphorylation of serine residues that may result in feedback inhibition of IR and IGF-IR downstream signaling or insulin resistance in diabetic models. Predictors of such biological responses focus on both the inhibition of IRS tyrosine phosphorylation and the ability of serine phosphorylated IRS proteins to bind and become phosphorylated by IR. Ser^{307} of IRS-1 has become particularly interesting since it lies adjacent to the PTB (protein tyrosine binding) domain, the region required for receptor binding (Aguirre et al., 2002). In diabetic models, JNK (activated by TNFα, anisomysin, and PI 3-kinase sensitive kinases) phosphorylates this site to inhibit IRS downstream signaling (Rui et al., 2001). In contrast, the PI 3-kinase sensitive kinase, Akt, has four consensus serine phosphorylation motifs in IRS-1, not including Ser^{307}, that when phosphorylated inhibits tyrosine de-

phosphorylation of IRS-1 resulting in prolonged IRS-1 mediated responses (Paz et al., 1999). The region containing Ser^{307} also does not appear to be a phosphorylation motif for other kinases reported to phosphorylate IRS-1, including MAPK and PI 3-kinase (De Fea & Roth, 1997). Further, the stress kinase p38 is an unlikely candidate in our model since IGF-I treatment does not enhance its activity in MCF-7 cells (data not shown). Interestingly, Rui and colleagues (Rui et al., 2001) have predicted that kinases other than just JNK may phosphorylate IRS-1 Ser^{307}. This conclusion was based on data showing TNFα stimulation of Ser^{307} phosphorylation is inhibited by PD98059. Although LY294002 blocked serine phosphorylation, it did not inhibit insulin activation of JNK after a 10 minute exposure time. It is unclear if JNK may be activated at later time-points in this model. However, since LY294002 co-treatment completely inhibited JNK activation by IGF-I and since IGF-I mediated Ser^{312} phosphorylation in our cells is also mediated by a PI 3-kinase sensitive kinase then JNK is the most likely sole mediator of this effect. Further, Taxol treatment both activated JNK and enhanced Ser^{312} phosphorylation of IRS-1 in our model.

To our knowledge, little is published regarding the subsequent biological effects of growth factor activation of JNK diabetic or cancer models, particularly looking at the combined effects of growth factors and chemotherapeutic agents in cancer models. These responses may be particularly relevant given that many solid tumors, including breast, prostate, lung and colon cancer, commonly overexpress IGF-IR. These tumors are also often treated with chemotherapeutic agents. Intuitively, one may anticipate that the serine phosphorylation of downregulatory sites such as Ser^{312} of IRS-1 and subsequent tyrosine dephosphorylation may be observed to a much lesser degree in cancer cells compared to normal cells. Our results indicate that phosphorylation of Ser^{312} is quite high in cancer cells. On the other hand, we did not

observe a very significant decrease in IRS-1 tyrosine phosphorylation using JNK overexpression approaches. Inhibition of PI 3-kinase in MCF-7 cells and JNK transfectants resulted in faster mobility of tyrosine phosphorylated IRS-1 indicating a reduction in serine phosphorylation. This data lead to uncertainty as to whether serine phosphorylation of IRS-1 actually inhibits its ability to serve as an IGF-IR substrate in breast cancer cells. We have not yet studied this interaction in our model. Somewhat in contrast to our model, Hemi and colleagues report that IRS-1 serine phosphorylation was enhanced by anisomycin, TNFα, and Smase (sphingomyelinse) treatment via phosphorylation ErbB2 and 3 in Fao hepatoma cells but that tyrosine phosphorylation of IRS-1 was inhibited by LY294002 (Hemi et al., 2002). Interestingly, JNK activity in response to the above treatments was not assessed in this paper but this data may implicate the complexity of PI 3-kinase signaling through both Akt and JNK (Figure 10). These findings also underline the relevance of crosstalk among stress and growth factor related pathways that may converge on JNK and may be directly applied to cancer models as therapeutic targets.

Clearly, the biological role(s) of JNK are controversial (see review by Minden and Karin (Minden & Karin, 1997)). Simple activation of JNK by any agonist does not imply the same biological response. JNK activity may either convey resistance or sensitivity to cellular stress (Fuchs et al., 1998; Okubo et al., 1998) or even proliferative responses (Bost et al., 1997). Some investigators have begun to study the effects of diverse stimuli on kinases upstream of JNK and differential regulation resulting from the various JNK isoforms derived from the three JNK genes, JNK1, JNK2 and JNK3 (Cuenda et al., 1997; Gupta et al., 1996) in attempts to understand the various biological responses associated with JNK activity. Our findings that IGF-I and stress treatments activate JNK to inhibit IGF-I signaling, make JNK an excellent target for cancer

treatment. Inducing JNK response may simultaneously enhance the efficacy of radio- and chemotherapy as well as attenuating IGF-IR responses in cancer models.

References Cited

Aguirre, V., Werner, E.D., Giraud, J., Lee, Y.H., Schoelson, S.E. & White, M.F. (2002). *Journal of Biological Chemistry*, **277,** 1531-1537.

Alblas, J., Slager-Davidov, R., Steenbergh, P.H., Sussenbach, J.S. & van der Burg, B. (1998). *Oncogene*, **16,** 131-139.

Bost, F., McKay, R., N., D. & Mercola, D. (1997). *Journal of Biological Chemistry*, **272,** 33422-33429.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. & Greenberg, M.E. (1999). *Cell*, **96,** 857-868.

Butler, A.A., Blakesley, V.A., Tsokos, M., Pouliki, V., Wood, T.L. & LeRoith, D. (1998). *Cancer Research*, **58,** 3021-3027.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. & Reed, J.C. (1998). *Science*, **282,** 1318-1321.

Chen, N., Nomura, M., She, Q.B., Ma, W.Y., Bode, A.M., Wang, L., Flavell, R.A. & Dong, Z. (2001). *Cancer Research*, **61,** 3908-3912.

Chen, Y.-R., Meyer, C.F. & Tna, T.-H. (1996). *Journal of Biological Chemistry*, **271,** 631-634.

Cuenda, A., Cohen, P., Buee-Scherrer, V. & Goedert, M. (1997). *EMBO Journal*, **16,** 295-305.

Cullen, K.J., Yee, D., Sly, W.S., Perdue, J., Hampton, B., Lippman, M.E. & Rosen, N. (1990). *Cancer Research*, **50,** 48-53.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M.E. (1997). *Cell*, **91,** 231-241.

De Fea, K. & Roth, R.A. (1997). *Journal of Biological Chemistry*, **272,** 31400-6.

Desbois-Mouthon, C., Blivet-Van Eggelpoel, M.-J., Auclair, M., Cherqui, G., Capeau, J. & Caron, M. (1998). *Biochemical and Biophysical Research Communications*, **243,** 765-770.

Dunn, S.E., Ehrlich, M., Sharp, N.J.H., Solomon, G., Hawkins, R., Baserga, R. & Barrett, J.C. (1998). *Cancer Research*, **58,** 3353-3361.

Fanger, G.R., Lassignal Johnson, N. & Johnson, G.L. (1997). *EMBO Journal*, **16,** 4961-4972.

Franke, T.F., Yang, S.-I., Chan, T.O., Datta, K., Kaziauskas, A., Morrison, D.K., Kaplan, D.R. & Tsichlis, P.N. (1995). *Cell*, **81,** 727-736.

Fuchs, S.Y., Fried, V.A. & Ronai, Z. (1998). *Oncogene*, **17,** 1483-1490.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R. & Cohen, P. (1997). *EMBO Journal*, **16,** 3563-3571.

Gooch, J.L., Van Den Berg, C.L. & Yee, D. (1999). *Breast Cancer Research and Treatment*, **56,** 1-10.

Gupta, S., Barrett, T., Whitmarsh, A.J., Cavanagh, J., Sluss, H.K., Derijard, B. & Davis, R.J. (1996). *EMBO Journal*, **15,** 2760-2770.

Hemi, R., Paz, K., Wertheim, N., Karasik, A., Zick, Y. & Kanety, H. (2002). *Journal of Biological Chemistry*, **277,** 8961-8969.

Hermanto, U., Zong, C.S. & Wang, L.H. (2000). *Cell Growth and Differentiation*, **12,** 655-64.

Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. (1993). *Genes Dev*, **7,** 2135-2148.

Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tsichlis, P.N. & Hay, N. (1997). *Genes & Development*, **11,** 701-713.

Kyriakas, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. & Woodget, J.R. (1994). *Nature(London)*, **369,** 156-159.

Lee, A.V., Gooch, J.L., Oesterrich, S., Guler, R.L. & Yee, D. (2000). *Molecular and Cellular Biology*, **20,** 1489-1496.

Logan, S.K., Falasca, M., Hu, P. & Schlessinger, J. (1997). *Molecular and Cellular Biology*, **17,** 5784-5790.

Lopez-Ilasaca, M., Li, W., Uren, A., Yu, J., Kazlauskas, A., Gutkind, J.S. & Heideran, M.A. (1997). *Biochemical and Biophysical Research Communications*, **232,** 273-277.

Miller, B.S., Shankavaram, U.T., Horney, M.J., Gore, A.C.S., Kurtz, D.T. & Rosenzweig, S.A. (1996). *Biochemistry*, **35,** 8769-8775.

Minden, A. & Karin, M. (1997). *Biochimica et Biophysica Acta*, **1333,** F85-F104.

Mitsuuchi, Y., Johnson, S.W., Selvakumaran, M., Williams, S.J., Hamilton, T.C. & Testa, J.R. (2000). *Cancer Research*, **60,** 5390-5394.

Monno, S., Newman, M.V., Cook, M. & Lowe, W. (2000). *Endocrinology*, **141,** 544-50.

Okubo, Y., Blakesley, V.A., Stannard, B., Gutkind, S. & Le Roith, D. (1998). *Journal of Biological Chemistry*, **273,** 25961-25966.

Paz, K., Liu, Y.-F., Shorer, H., Hemi, R., LeRoith, D., Quan, M., Kanety, H., Seger, R. & Zick, Y. (1999). *Journal of Biological Chemistry*, **274,** 28816-28822.

Peyrat, J.P., Bonneterre, J., Jammes, H., Beuscart, R., Hecquet, B., Djiane, J., Lefebvre, J. & Demaille, A. (1990). *Journal of Steroid Biochemistry and Molecular Biology*, **37,** 823-827.

Potapova, O., Gorospe, M., Dougherty, R.H., Dean, N.M., Gaarde, W.A. & Holbrook, N.J. (2000). *Molecular and Cellular Biology*, **20,** 1713-1722.

Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M.J., Gjerset, R. & Mercolas, D. (1995). *Journal of Biological Chemistry*, **272,** 14041-14044.

Resnik, J.L., Reichart, D.B., Huey, K., Webster, N.J.G. & Seely, B.L. (1998). *Cancer Research*, **58,** 1159-1164.

Rosette, C. & Karin, M. (1996). *Science*, **274,** 1194-7.

Rui, L., Aguirre, V., Kim, J.K., Shulman, G.I., Lee, A., Corbould, A., Dunaif, A. & White, M.F. (2001).

She, Q.B., Chen, N., Bode, A.M., Flavell, R.A. & Dong, Z. (2002). *Cancer Research*, **62,** 1343-1348.

Singer, C., Rasmussen, A., Smith, H.S., Lippman, M.E., Lynch, H.T. & Cullen, K.J. (1995). *Cancer Research*, **55,** 2448-2454.

Sluss, H.K., Barrett, T., Derijard, B. & Davis, R.J. (1994). *Molecular and Cellular Biology*, **14,** 8376-84.

Smith, D.B. & Johnson, K.S. (1988). *Gene*, **67,** 31-40.

Van Den Berg, C.L., Cox, G.N., Stroh, C.A., Hilsenbeck, S.G., Weng, C., McDermott, M.J., Pratt, D. & Yee, D. (1997). *European Journal of Cancer*, **33,** 1108-1113.

Wang, T.-H., Eang, H.-S., Ichijo, H., Giannakakou, P., Foster, J.S., Fojo, T. & Wimalasena, J. (1998). *Journal of Biological Chemistry*, **273,** 4928-3936.

Westwick, J.K., Weitzel, C., Minden, A., Karin, M. & Brenner, D.A. (1994). *Journal of Biological Chemistry*, 26396-26401.

Whitmarsh, A.J., Shore, P., Sahrrocks, A.D. & Davis, R.J. (1995). *Science*, **269,** 403-407.

Footnotes

* This work was supported in part by Susan G. Komen Foundation (to C.L.V.D.B.); the Burroughs Wellcome Fund/American Foundation for Pharmaceutical Education (to C.L.V.D.B.); and Public Health Service Grant CA89288A awarded by the National Cancer Institute (to C.L.V.D.B.); and United States Army Medical Research and Command Grant DAMD17-99-1-9142 (to C.L.V.D.B). The content of this information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

§ To whom correspondence should be addressed: Carla Van Den Berg, School of Pharmacy, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Campus Box C238, Denver, CO 80262. Tel.: (303) 315-5948; Fax: (303) 315-4630; E-mail: carla.vandenberg@UCHSC.edu.

Acknowledgements: We would like to thank Heather Ferguson for thoughtful and critical review of this manuscript.

Figure Legends

Figure 1. Taxol and Taxotere treatment increases JNK activity. ***A.*** Cells were treated with either doxorubicin (not shown graphically), Taxol (■), or Taxotere (▲) at concentrations indicated and harvested in a time-dependent fashion. UV (50 J/M^2) was used as a positive control for JNK activation in MCF-7 cells. Analyses of phosphorylated c-$Jun_{(1-79)}$ resulting from *in vitro* kinase assays are shown. ***B.*** The graph shows the combined results of two independent experiments (points: mean; bars: range).

Figure 2. JNK activity is dramatically increased with IGF-I treatment. MCF-7 and T47D breast cancer cells were treated with IGF-I (50 ng/ml) in a time-course experiment. ***A.*** Analyses of phosphorylated c-$Jun_{(1-79)}$ resulting from *in vitro* kinase assays of MCF-7 cells are shown. UV (50 J/M^2) was used as a positive control for JNK activation in MCF-7 cells. ***B.*** The graph shows the combined results of at least two independent experiments (mean ± range).

Figure 3. JNK activation by IGF-I is PI 3-kinase dependent in MCF-7 cells. ***A.*** T47D and MCF-7 breast cancer cells were pretreated with LY294002 or wortmannin as indicated for 40 minutes prior to IGF-I treatment for 30 and 60 minutes, respectively. Analyses of phosphorylated c-$Jun_{(1-79)}$ resulting from *in vitro* kinase assays are shown. ***B.*** The graph shows the combined results of at least two independent experiments (mean ± range).

Figure 4. Co-treatment IGF-I and chemotherapy results in additive effects on JNK. ***A.*** JNK activity was measured by exposing cells to Taxol (0.25 μM) and IGF-I (50 ng/ml) simultaneously. Cells were exposed to wortmannin (100 nM) 40 minutes at concentrations indicated prior to IGF-I treatment. Western blot analyses of kinase reactions were performed to compare JNK protein levels for each treatment. ***B.*** Graphical representation of above kinase assays showing the combined results of at least two independent experiments (mean ± range). ***C.***

The effect of IGF-I co-treatment with Taxotere (1 μM for 2 hours) and UV (50 J/M^2 for 50 minutes) on JNK activity was tested by exposing cells to each treatment until time of maximal response of each treatment. Western blot analysis was performed to compare JNK expression in each treatment. **D.** Each graph shown represents three independent experiments (mean ± s.e.m.).

Figure 5. GST-Akt peptide substrate is phosphorylated by IGF-I treated MCF-7 cell lysates. GST-Akt specific substrate (GST-GSK) was developed as described in Methods. ***A.*** Cells were treated with IGF-I 50 ng/ml and at the indicated times as described in Methods. Phosphorylated GST-GSK product resulting from *in vitro* kinase assays was detected and measured. The graph shown represents three independent experiments (mean ± s.e.m.) ***B.*** MCF-7 cells were either pre-incubated in 50μM LY294002 for 40 minutes prior to IGF-I treatment or treated with IGF-I alone, as indicated. Phosphorylated GST-GSK was analyzed using SDS-PAGE and autoradiography.

Figure 6. Overexpression of Mt Akt or Wt JNK enhanced Taxol induced apoptosis and reduced IGF-I survival effects. ***A.*** Parental cells and Wt or Mt Akt transfectants were plated in chamber slides and treated as described in Methods. Forty-eight hours after exposure, cells were stained with Hoechst 33258 for analysis of apoptotic nuclei. ***B.*** Parental MCF-7 cells and Wt or Mt JNK transfectants were tested in the same fashion as Akt transfectants above. Bars are the average of three independent experiments for both Akt and JNK experiments. Error bars show 95% C.I. around the mean.

Figure 7. IGF-I inhibits Taxol-mediated apoptosis while Wt JNK overexpression enhances PARP cleavage induced by Taxol. MCF-7 cells were plated overnight then serum starved. Taxol treatment was added at increasing concentrations (0.01-0.04 μM, as indicated) with or

without IGF-I co-treatment (50 ng/ml), as indicated. 48 hours after treatment, cells were lysed and PARP cleavage was measured by Western blot analysis of parental and cleaved PARP fragments. The presence of an 85 kD cleavage fragment indicates induction of apoptosis. ***A.*** IGF-I inhibited PARP cleavage at Taxol concentrations less than 0.04 μM/ml. ***B.*** Cells were treated in the same fashion as above but some cells were co-treated with or without IGF-I and LY294002 (50 μM) for 40 minutes as indicated. ***C.*** Transient transfection of MCF-7 cells using empty vector and a Myr Akt containing plasmid was performed and then cells were treated as described above. ***D.*** MCF-7 cells and stable Wt JNK transfectant cells were treated in the same fashion described above. PARP cleavage was analyzed by Western blot using a PARP primary antibody.

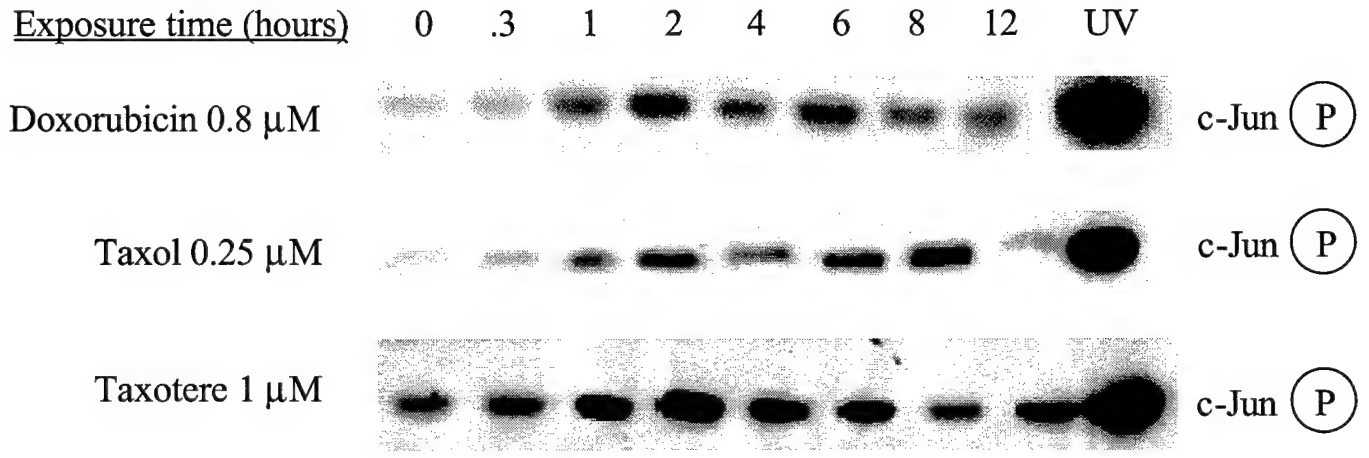
Figure 8. JNK overexpression inhibits anchorage independent growth and IGF-I stimulated anchorage independent growth. Parental MCF-7 cells and stable Wt and Mt JNK transfectant cells were suspended cells in 5% CSS and 0.8% Seaplaque agarose. Cells were seeded at 30,000 per dish and grown for 9 days. IGF-I (50 ng/ml) was added as indicated. Each sample was grown in triplicate and colonies were counted within the same surface area.

Figure 9. IGF-I and Taxol treatments lead to Ser^{312} phosphorylation of IRS-1 and IGF-I effects were blocked by PI 3-kinase inhibition. MCF-7 cells were plated overnight then serum starved. Some samples were pre-treated with LY294002 100 μM for 40 minutes, as indicated. Cells were treated with IGF-I (50 ng/ml) or Taxol (0.25 μM) for the duration of time indicated. All cells were lysed at the indicated times, lysates were cleared, and IRS-1 was immunoprecipitated using a total IRS-1 antibody. Ser^{312} phosphorylation was measured using a rat Ser^{307} specific antibody, which specifically cross-reacts with human phosphoserine 312. ***A.*** Phosphorylation of IRS-1 Ser^{312} in IGF-I treated cells and the effect of PI 3-kinase inhibition

with LY294001 was assessed by Western blot analysis. ***B.*** Phosphorylation of IRS-1 Ser^{312} in Taxol (0.25 μM) treated MCF-7 cells assessed using methods described above. ***C.*** MCF-7 cells were transiently transfected as described in Methods. Ser^{312} phosphorylation of mock transfected cells was compared to Wt JNK transfectants. ***D.*** Tyrosine phosphorylation of IRS-1 was measured in parental MCF-7 cells and Wt JNK transfectants using an anti-phosphotyrosine specific antibody for Western blot analysis.

Figure 10. IGF-I activates JNK, JNK then inhibits IGF-IR mediated signaling by serine phosphorylation of IRS-1. Our data support the proposed model where IGF-I activation of JNK leads to a negative feedback effect on IGF-IR signaling. This effect is observed when JNK is activated downstream of PI 3-kinase, and subsequently, activated JNK phosphorylates Ser^{312} of IRS-1 to inhibit IGF-IR responses.

A.


Exposure time (hours)
0
.3
1
2
4
6
8
12
UV
Doxorubicin 0.8 µM
c-Jun (P)
Taxol 0.25 µM
c-Jun (P)
Taxotere 1 µM
c-Jun (P)

B.

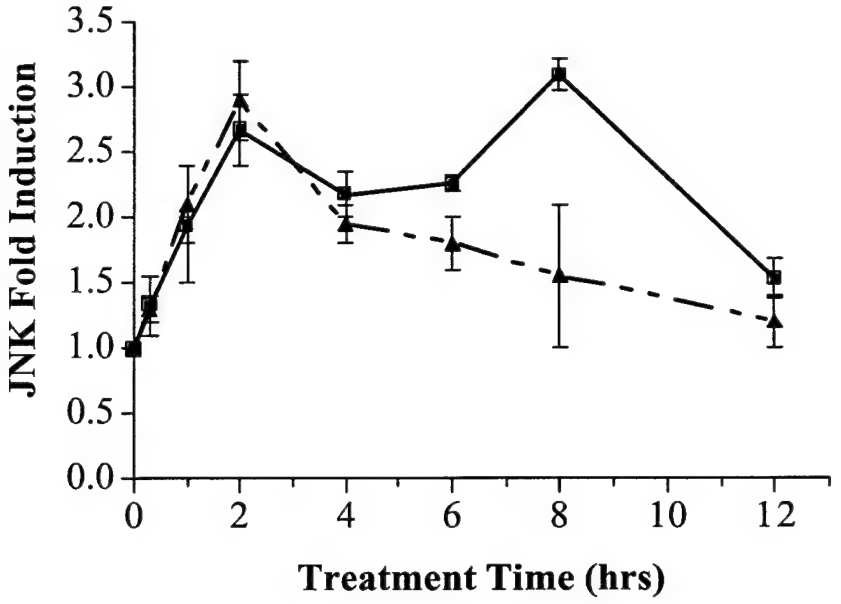

JNK Fold Induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
Treatment Time (hrs)

Figure 1

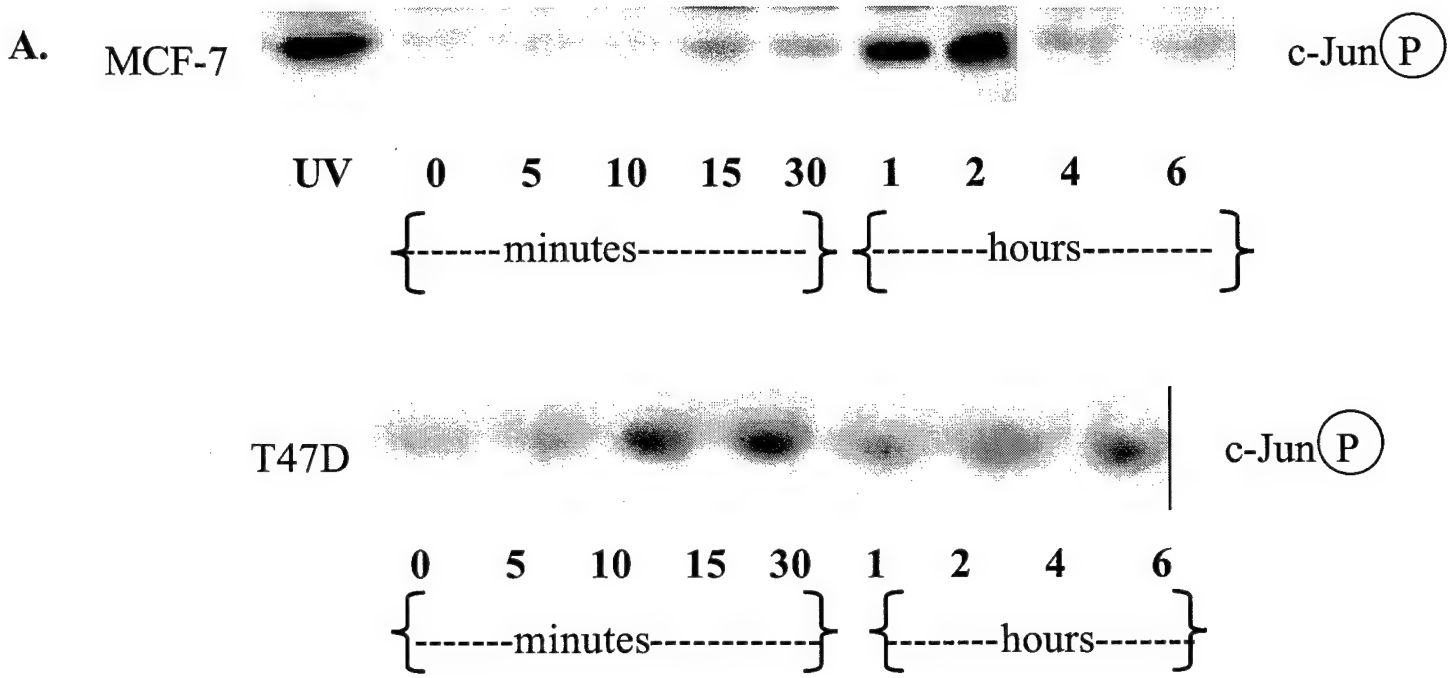

A.
MCF-7
c-Jun P
UV 0 5 10 15 30 1 2 4 6
minutes
hours
T47D
c-Jun P
0 5 10 15 30 1 2 4 6
minutes
hours
IGF-I Exposure Time

Figure 2.A

B.

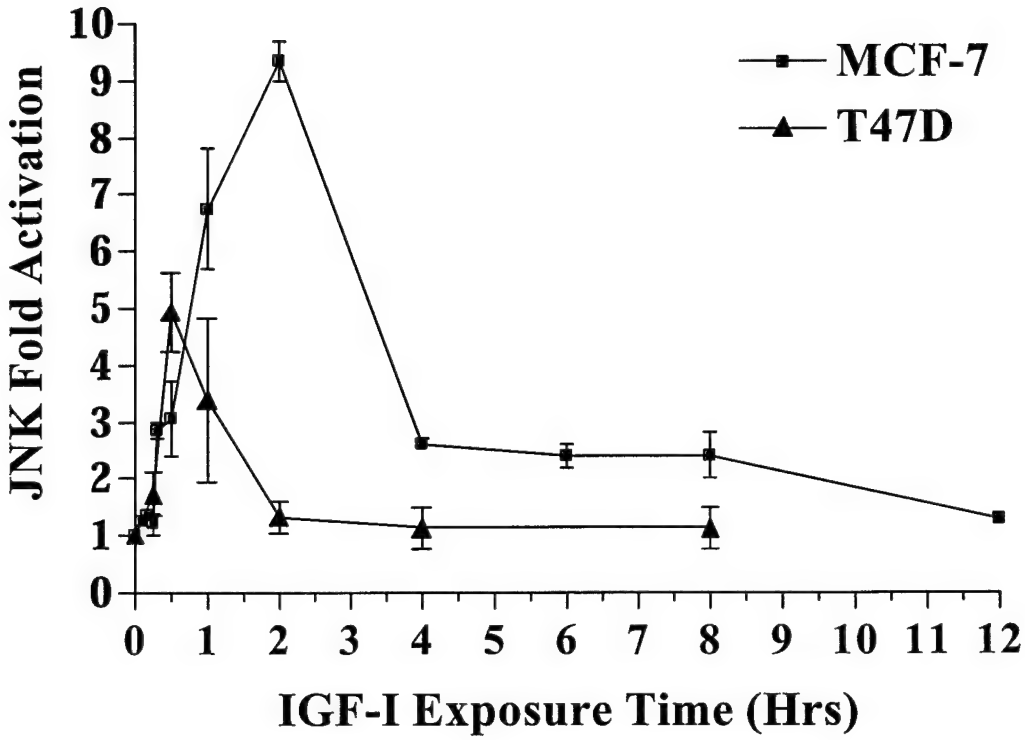
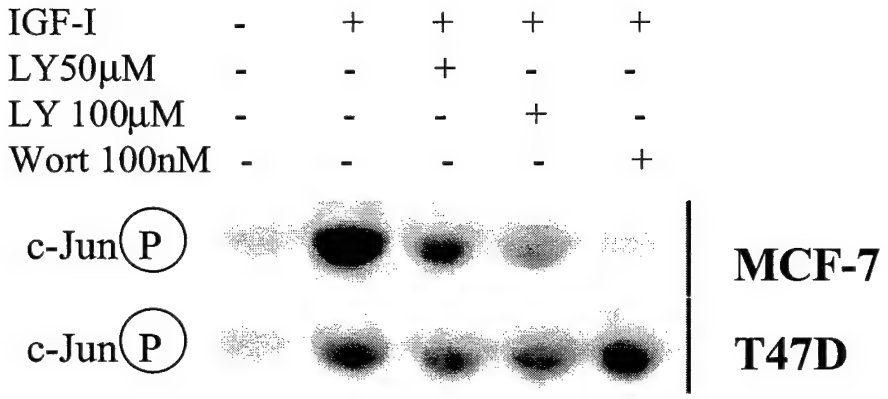

JNK Fold Activation
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
IGF-I Exposure Time (Hrs)
MCF-7
T47D

Figure 2.B

A.


IGF-I - + + + +
LY50μM - - + - -
LY 100μM - - - + -
Wort 100nM - - - - +
c-Jun P
c-Jun P
MCF-7
T47D

B.

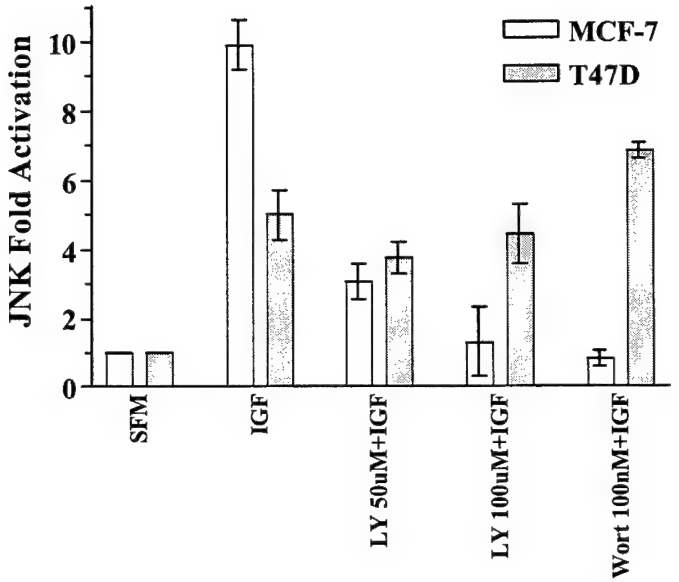
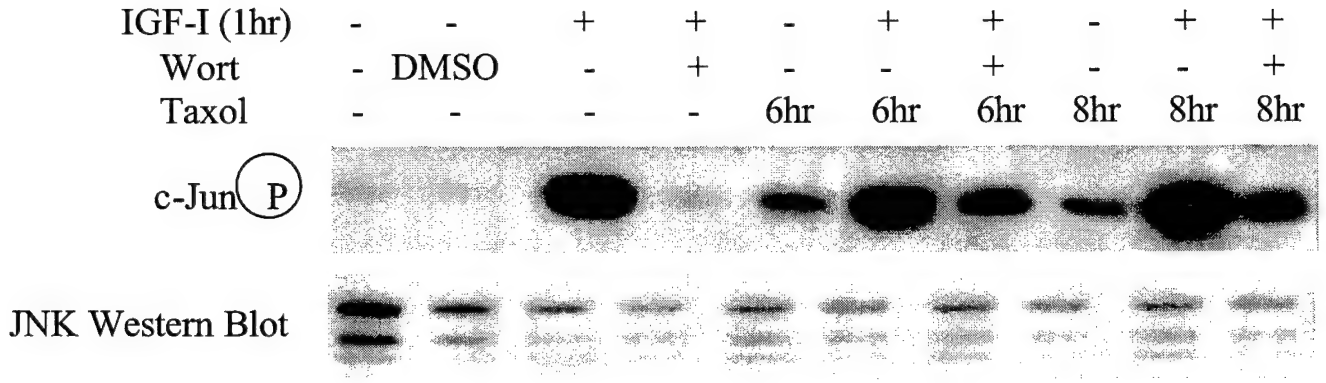

JNK Fold Activation
0
2
4
6
8
10
MCF-7
T47D
SFM
IGF
LY 50uM+IGF
LY 100uM+IGF
Wort 100nM+IGF

Figure 3

A.



Co-treatment with IGF-I 1 hr

B.

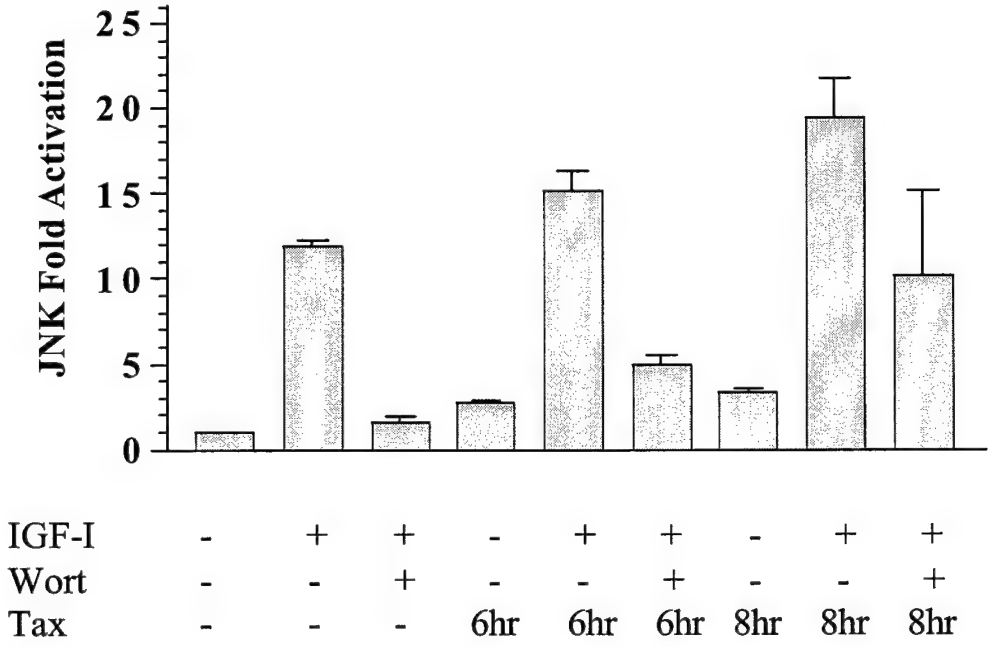
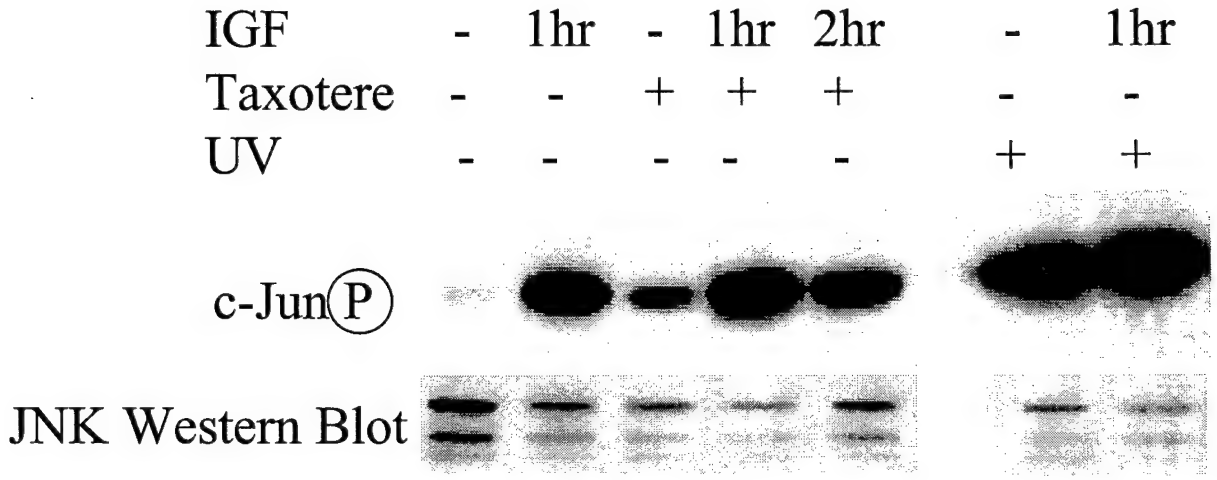


Figure 4.A and B

C.



D.

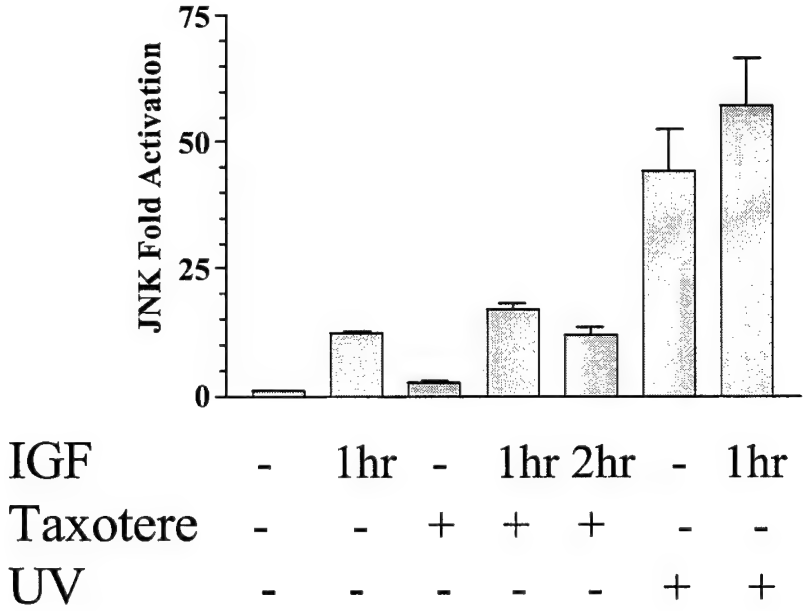
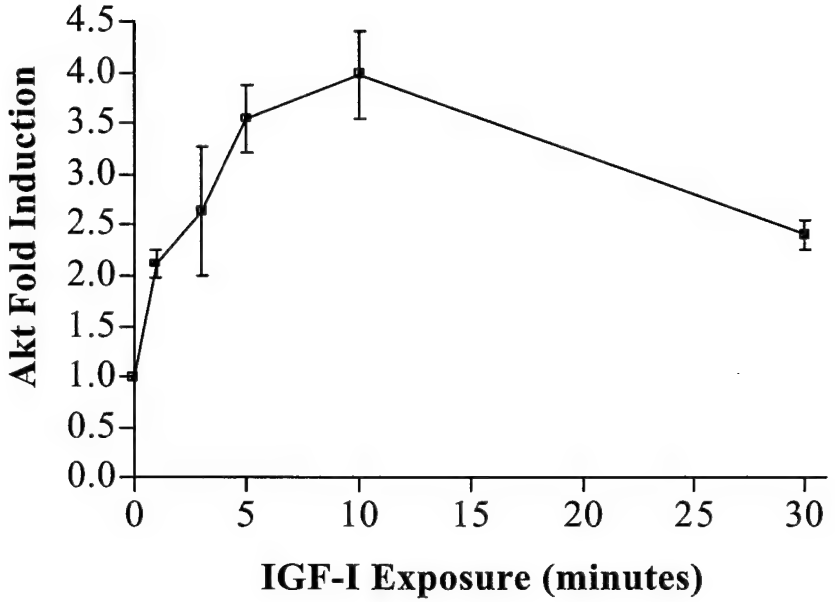


Figure 4.C and D

A.

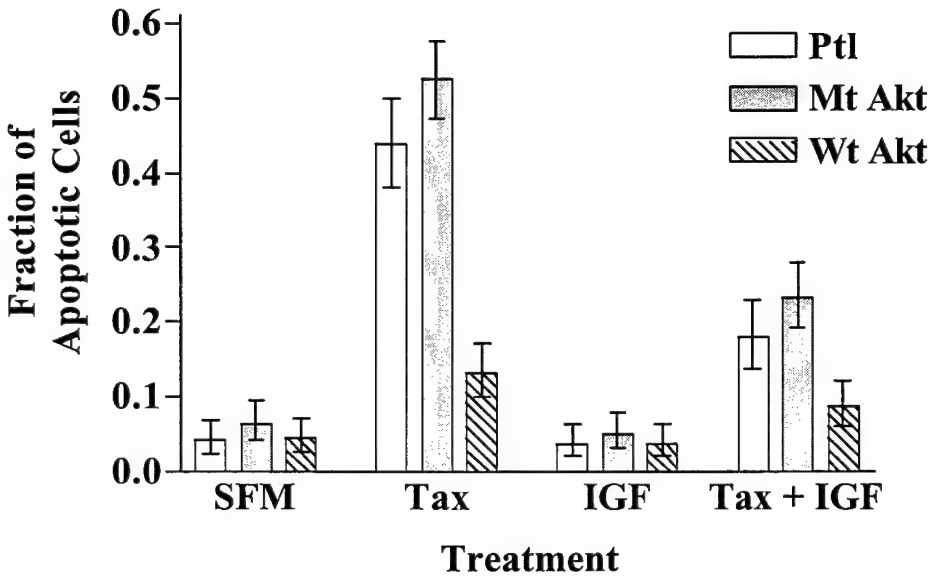


B.

IGF-I	-	3min	3min	5min	5min
LY	-	-	+	-	+
Fold change:	1.0	2.8	1.9	2.9	1.3

Figure 5

A.


Fraction of Apoptotic Cells
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SFM
Tax
IGF
Tax + IGF
Treatment
Ptl
Mt Akt
Wt Akt

B.

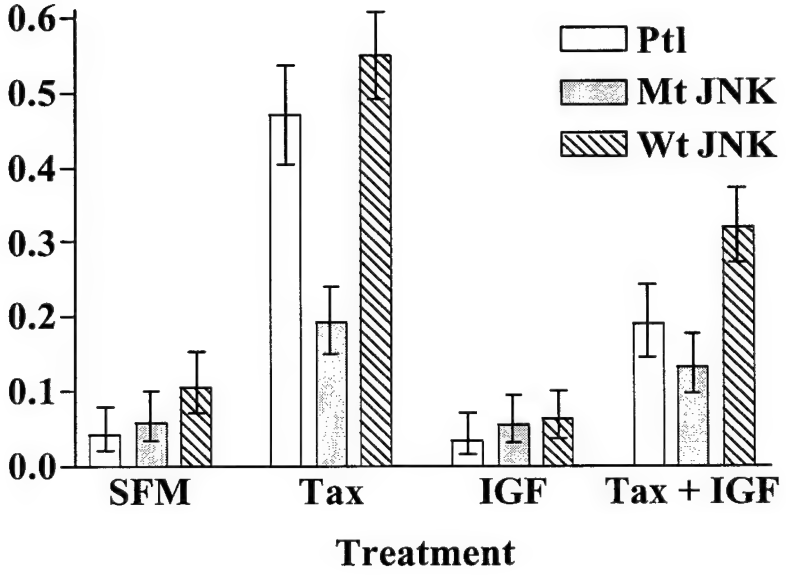

0.6
0.5
0.4
0.3
0.2
0.1
0.0
SFM
Tax
IGF
Tax + IGF
Treatment
Ptl
Mt JNK
Wt JNK

Figure 6

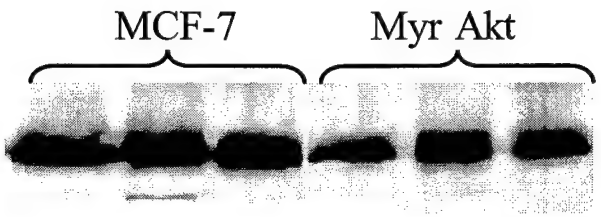
A.

Taxol	-	0.01	0.01	0.02	0.02	0.04	0.04
IGF-I	-	-	+	-	+	-	+

B.

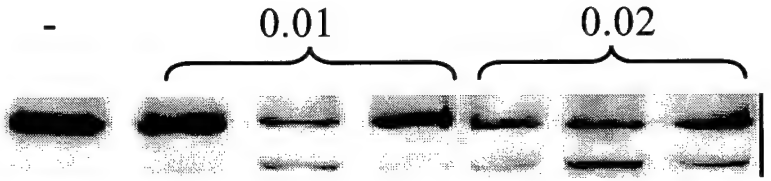
IGF-I	-	-	+	-	+
Taxol	-	0.02	0.02	0.02	0.02
LY	-	-	-	+	+

C.



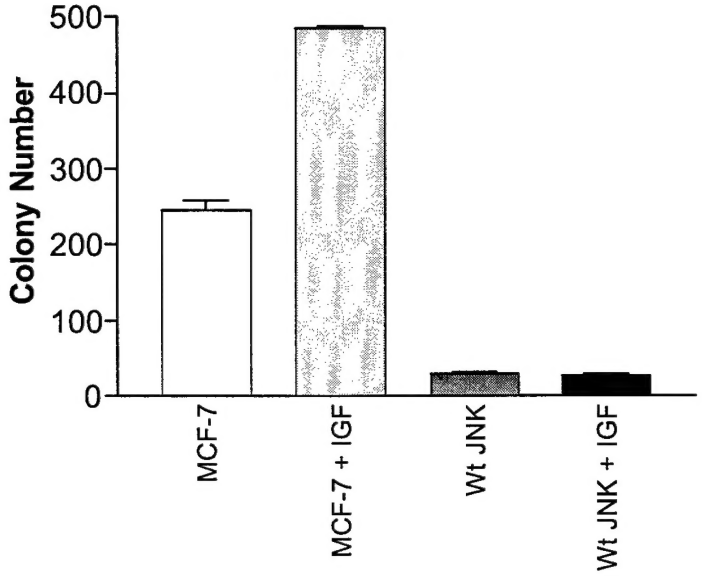
Taxol	-	0.02	0.02	-	0.02	0.02
IGF-I	-	-	+	-	-	+

D.



IGF-I	-	-	-	+	-	-	+
MCF-7	+	+	-	-	+	-	-
Wt JNK	-	-	+	+	-	+	+

Figure 7


Colony Number
500
400
300
200
100
0
MCF-7
MCF-7 + IGF
Wt JNK
Wt JNK + IGF
Treatment Group

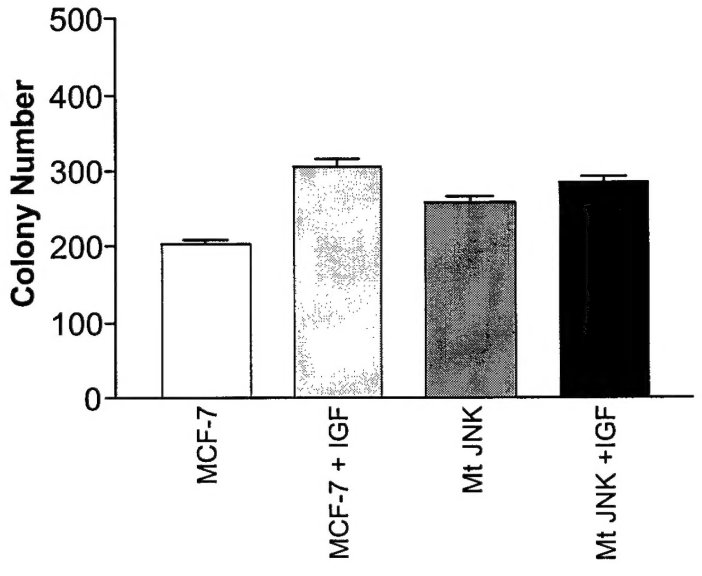
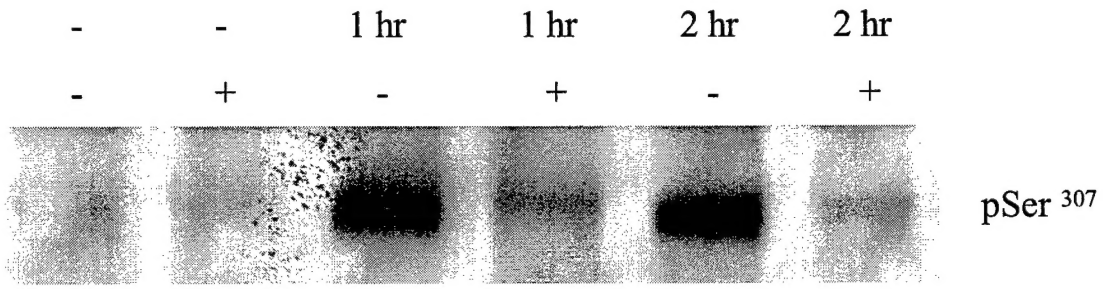

Colony Number
500
400
300
200
100
0
MCF-7
MCF-7 + IGF
Mt JNK
Mt JNK +IGF
Treatment Group

Figure 8

A.



B.

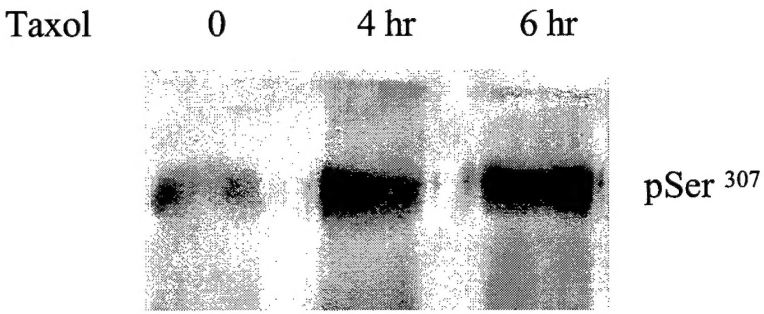
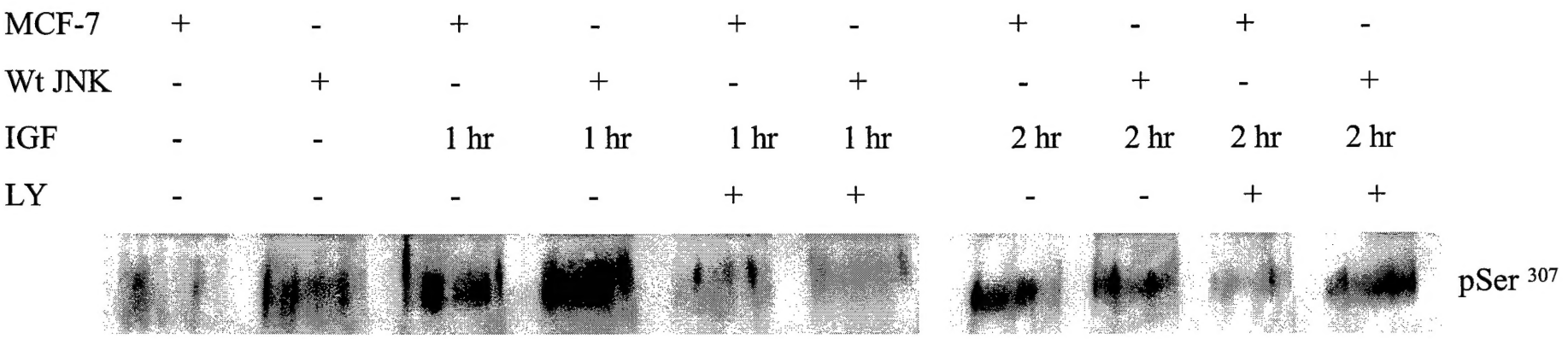


Figure 9.A and B

C.



D.

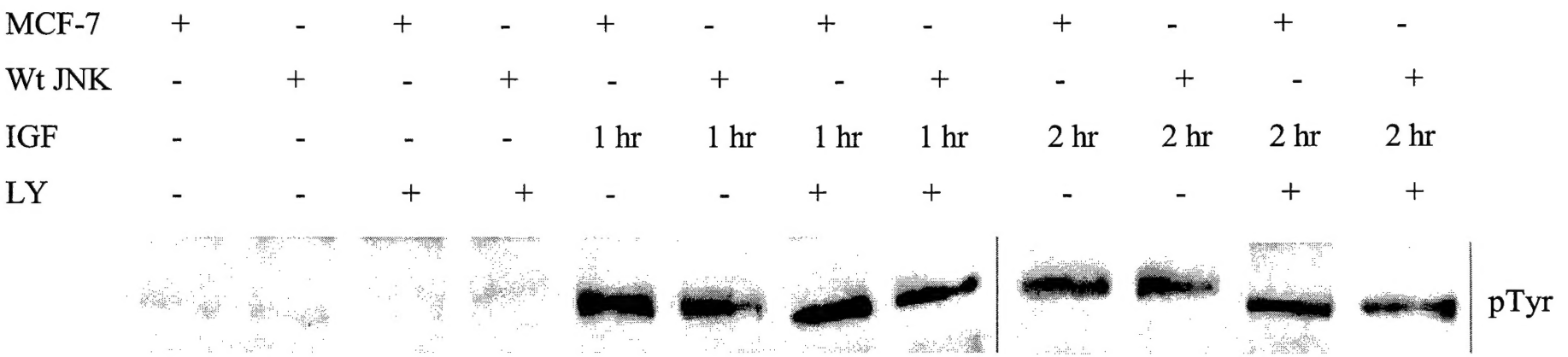


Figure 9.C and D

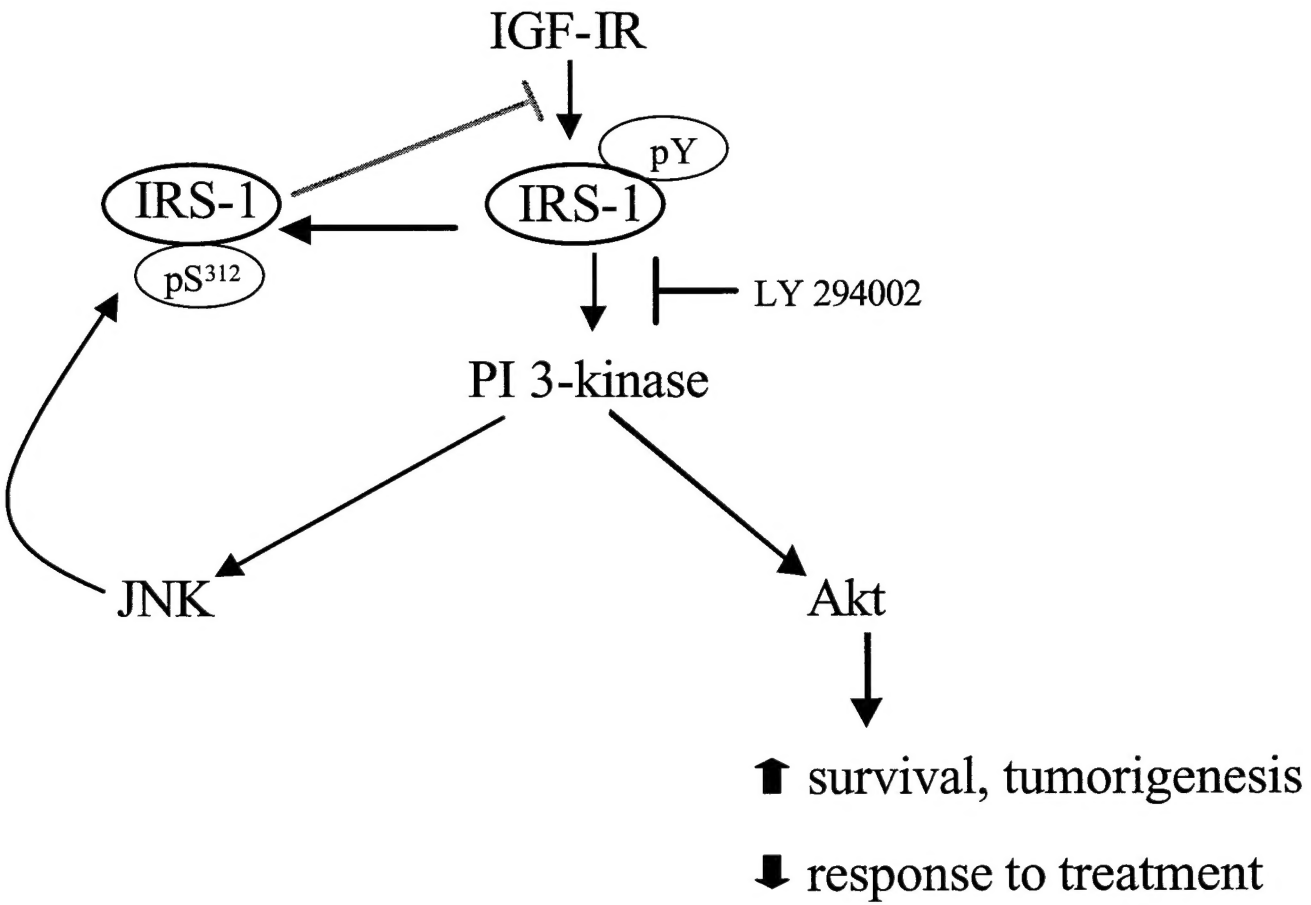

IGF-IR
pY
IRS-1
IRS-1
pS312
LY 294002
PI 3-kinase
JNK
Akt
survival, tumorigenesis
response to treatment

Figure 10